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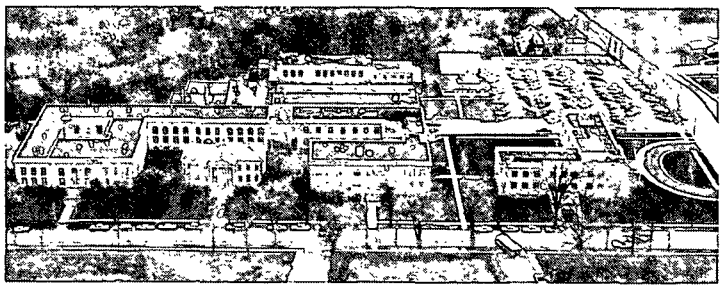

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PREPARATION OF VIABLE PROTOPLASTS FROM SUSPENSION-CULTURED LOBLOLLY PINE (*PINUS TAEDA*) CELLS AND SUBSEQUENT REGENERATION TO CALLUS

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PREPARATION OF VIABLE PROTOPLASTS FROM SUSPENSION-CULTURED LOBLOLLY PINE (PINUS TAEDA) CELLS AND SUBSEQUENT REGENERATION TO CALLUS

R. D. Teasdale[1] and E. Rugini[2]

ABSTRACT

Protoplasts were prepared from exponentially growing cell suspension cultures derived from Pinus taeda seedlings. The cell suspension was derived from callus that had been initiated on solid Murashige and Skoog medium supplemented with 2 mg/L 2,4-D and maintained in liquid LM medium (D.C. Verma et al., Proceedings of the 5th International Congress of Plant Tissue and Cell Culture, Tokyo, Japan, 1982. p. 59-60). Evaluation of a range of enzyme formulations showed that almost all cells could be converted to viable protoplasts in 9 hours with the combination of 0.5% each of Onozuka R-10, Macerozyme R-10, Hemicellulase, Driselase, and bovine serum albumin in osmoticum (0.4M mannitol, 0.2M sorbitol, 1.5mM $CaCl_2$, 0.7mM KCl). Protoplast purification required only simple washing, which was achieved without damaging centrifugation. When cultured in LM medium supplemented with 1.5mM $CaCl_2$, 10mM arginine, 10mM glutamine and 5mM asparagine, cell wall formation was observed after 48 hours. The cultured protoplasts produced numerous colonies in 3 weeks, which continued to grow in liquid medium.

Keywords

cell suspension culture

protoplast preparation

protoplast culture

Pinus taeda

protoplast regeneration

conifer protoplasts

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INTRODUCTION

The considerable benefits to plant science offered by protoplast techniques have been described frequently and at length, and many examples of successful protoplast preparation and regeneration exist [2,10]. However, little success has been obtained for cell or callus formation from protoplasts of gymnosperms generally [1,5], and no reports have been made of the preparation of viable protoplasts from Pinus taeda. This paucity of success is presumably related to the difficulties encountered in maintaining continuous liquid cultures of conifers due to nutritional problems. With forest species of long generation time in particular, the potential of in vitro genetic improvement techniques such as transformation, somatic hybridization, and clonal selection cannot be overestimated. Furthermore, protoplasts offer potential in numerous metabolic and physiological studies, e.g., the uptake of nutrients in the absence of such complications as diffusional barriers and cell wall adsorption [8,9], and isolation of subcellular organelles [7]. Protoplasts employed in any of these studies ideally should be readily and quantitatively produced in sterile form, be easily purified from unwanted materials (vacuolated or differentiated cells), be genetically and nutritionally uniform, and finally, in order to be free of developmental variation, should consist wholly of nondifferentiated meristematic cells. The availability of a rapidly growing and apparently meristematic suspension culture of loblolly pine cells with the recently formulated LM medium [11] offered the possibility of meeting this ideal and encouraged us to evaluate this culture system for use in protoplast preparation.

MATERIALS AND METHODS

Hypocotyls from a two-week-old Pinus taeda seedling were surface sterilized (50% Hilex containing 5.25% sodium hypochlorite plus 1% Tween 20 for 20 minutes) and

placed on solid Murashige and Skoog (MS) medium [6] containing 0.7% agar and 2 mg 2,4-dichlorophenoxyacetic acid/L. After subculturing for 15 weeks at 5 week intervals the callus was transferred to liquid LM medium and incubated with shaking at 23°C in the light with a photoperiod of 16 hours (3000 lux cool white fluorescent lamps). This medium is characterized by high Mg^{2+}, low Ca^{2+}, and high levels of most mineral micronutrients. The resultant suspension culture consisted of small friable cell clusters. It was maintained by transferring 5 mL of culture in 50 mL of fresh medium every 14 days. To obtain a growth curve, cultured tissue was broken up by stirring on a 500-μm metal sieve, then cell clusters were collected on a 100-μm metal sieve, washed, and resuspended in fresh medium at a density of ca. 5 μL packed cell volume per mL. Inocula were grown in 1-mL volumes using multiwell trays (24-well, Costar disposable labware) shaken in the dark at 23°C. Dry weight measurements were made in quadruplicate at required time intervals by filter harvesting cells on tared glass fiber discs, washing with ca. 10 mL bidistilled water, and drying overnight at 80-90°C before weighing to a precision of 0.1 mg.

Protoplasts were prepared from tissue collected on a 125-μm sieve and preconditioned for 90 minutes in osmoticum (0.4M mannitol, 0.2M sorbitol, 1.5mM $CaCl_2$, 0.7mM KCl, pH 5.8). The enzyme solutions specified in Table 1 were prepared in osmoticum and filter sterilized after centrifuge-clarification and pH readjustment where necessary. Enzyme combination studies were performed in multiwell trays (12 well) by digesting quadruplicate samples of 100 μL of tissue in 2 mL of appropriate enzyme solutions. Estimates of protoplast yield were made by direct microscopic examinations and scoring of 6 fields by two observers. Protoplast viability was indicated by exclusion of the vital stain Evan's blue [4], and again 6 fields were scored by two observers. Protoplasts used for culture studies were prepared by the best enzyme treatment (combination F, Table 1). Following enzyme digestion, 10 mL

of protoplast suspension was allowed to naturally sediment in a conical 50-mL centrifuge tube after which the supernatant was removed. The approximately 0.5 mL of protoplast pellet was resuspended in 10 mL of fresh osmoticum, and the procedure was repeated. After 5 washes the protoplasts were suspended at a density of 5×10^4/mL in an equivolume mixture of osmoticum and LM medium, supplemented with 1.5 mM $CaCl_2$, 10mM arginine, 10mM glutamine, and 5mM asparagine, which were beneficial for growth of suspension culture (data not shown). A 5-mL aliquot of this protoplast suspension was cultured at 23°C for 3 days with gentle shaking under low light intensity (300 lux fluorescent lamps) for 16 hours per day in a 5.5-cm petri dish with a gas-permeable bottom (Heraeus petriperm). With other conditions held constant, the osmolarity was gradually reduced by replacing half the supernatant volume with modified LM medium every 3 days for a further 12 days. At these times samples were also taken for microscopic examination of cell wall regeneration, by use of fluorescent staining with Calcofluor White M2R (3), and of subsequent cell division. At 15 days a portion of the cells was transferred to solid medium (modified LM, 0.8% agar, 2 mg 2,4-dichlorophenoxyacetic acid acid/L).

The enzymes Onozuka R-10 and Macerozyme R-10 were obtained from Yakult Pharmaceutical Industry Ltd., Nishinomiya, Japan; Hemicellulase and Bovine Serum Albumin (Cohn fraction V) from Sigma Chemical Company, St. Louis, MO, U.S.A., and Driselase from Kyowa Hakko Kogyo Company, Tokyo, Japan. All other chemicals employed were of analytical reagent grade or higher.

RESULTS AND DISCUSSION

Loblolly pine suspension culture used herein was of high quality, dominated by small, round, nonvacuolated cells filled with many starch granules. The growth curve (Fig. 1a and 1b) shows that these cells grow exponentially, with a doubling

time of 4.9 ± 0.2 days. Extrapolation of the data to zero time coincides with the inoculum size, indicating that all introduced cells grow without extensive conditioning of the medium, characteristics well suited to protoplast preparation.

The results in Table 1 indicate that cellulase, pectinase, and hemicellulase are all required for optimal cell wall degradation. The relative proportions of these enzymes must be balanced to the composition of the cell wall, since any gross imbalance results in unnecessarily high levels of deleterious enzymes that are known to contaminate all these preparations, without an acceleration of the rate of cell wall decomposition. Thus, it was necessary to find an enzyme combination in which viability was optimized rather than yield. The protoplast yields after 4 and 7 hours of digestion (Table 1) show that treatments A and F are highly effective for removal of cell walls; indeed both completely converted all cells to protoplasts in 9 hours (data not shown). However, the viabilities of the protoplasts obtained by these treatments differ. The results of Table 1 show that protoplasts produced by treatment F are almost all viable, whereas those from treatment A are not. Since combination F differs from A solely in the inclusion of bovine serum albumin (BSA), the latter presumably acts as a competitive substrate for contaminating proteases, thereby protecting membrane proteins, although other conceivable roles may be responsible.

Cell wall regeneration at ca. 48 hours of culture is shown by Calcofluor staining in Fig. 2A. A general deviation from spherical shape was evident throughout the culture at this time. After approximately 10 days of liquid culture, cell division had clearly occurred, as shown in Fig. 2B. After 2 to 3 weeks numerous colonies (10 + 3% of protoplasts present) developed to the stage where they were visible to the naked eye (Fig. 2C), and these continued to grow in liquid medium to yield callus. Growth on solid medium was also attempted, but cultures transferred

to agar at the small colony stage (15-30 days) grew only slowly, and culture death was not infrequent. Culture in liquid media has therefore been adopted by us as the most reliable and effective practice. While essentially all protoplasts prove intact with a viability stain and also regenerate cell walls, many do not develop further. In our view that is not an indication of some deficiency of the initial protoplast product, but is a later effect associated with the difficulty of growth of small cellular colonies which is not confined to protoplast-derived cells. When exponentially growing cell suspensions are finely sieved (100-500 μm diameter), they will not grow in liquid medium if the inoculum density is less than 0.05 mg (dry weight)/mL. Resolution of this problem and that of growth on solid medium awaits the outcome of wider nutritional and inoculum density studies currently in progress.

One notable feature of this protoplast preparation is the absence of centrifugation steps which have become standard for washing and purification. The well-defined cells employed here have a particularly high density due to the abundance of starch granules and absence of vacuoles. Consequently the protoplasts obtained from these cells sediment naturally in a short time, permitting gentle and simple washing while avoiding damaging centrifugation procedures.

The use of an exponentially growing culture provides cells which presumably are more readily protoplasted, since secondary cell wall formation is minimized. The particular mineral nutrient formulation of the LM medium may also affect the cell wall composition, so that the enzyme combination found appropriate in this study may require adjustment for use with loblolly pine cells cultured under different conditions. The few and gentle manipulations used in this procedure increase the convenience of preparation and are considered to contribute to the high yield of viable protoplasts. We therefore recommend that loblolly pine protoplasts should be

prepared from exponentially growing cells in suspension culture, and that attention be given to the formulation of an enzyme combination which gives high protoplast viability. It is expected that the particular procedure described here will prove useful for a variety of metabolic, genetic, and development studies with loblolly pine, especially those requiring large quantities of uniform protoplasts.

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TABLE 1

EFFECT OF ENZYME COMBINATIONS ON PROTOPLAST YIELD AND VIABILITY.

	Enzyme Combination[a] (%w/v)					Protoplast Yield (%)		Protoplast Viability
	Oz	Mz	Hs	Ds	B	At 4 hours	At 7 hours	(%) at 9 hours
A	0.5	0.5	0.5	0.5	-	70 ± 17	93 ± 2	89 ± 2
B	1.0	0.5	0.5	-	-	24 ± 19	59 ± 16	89 ± 2
C	0.5	2.0	2.0	-	-	1 ± 1	5 ± 5	73 ± 5
D	2.0	0.5	-	-	-	47 ± 25	78 ± 7	88 ± 3
E	2.0	-	0.5	-	-	8 ± 5	23 ± 13	58 ± 8
F	0.5	0.5	0.5	0.5	0.5	74 ± 16	91 ± 4	99.7 ± 0.4

[a]Abbreviations used: Oz, Onozuka R-10 (cellulase); Mz, Macerozyme R-10 (pectinase); Hs, hemicellulase; Ds, driselase; B, bovine serum albumin.

Figure 1. (a) A growth curve of mean dry weight (with standard deviations) plotted against time. This was obtained by growing 1-mL volumes of cell suspension in multiwell trays and filter harvesting quadruplicate samples at 3-4 day intervals as shown.

(b) The data of (a) replotted using the natural logarithm of harvest dry weights. The linearity of the plot shows that growth is exponential. Extrapolation of the data to the initial inoculum value (t=0) indicated that all introduced cells grow.

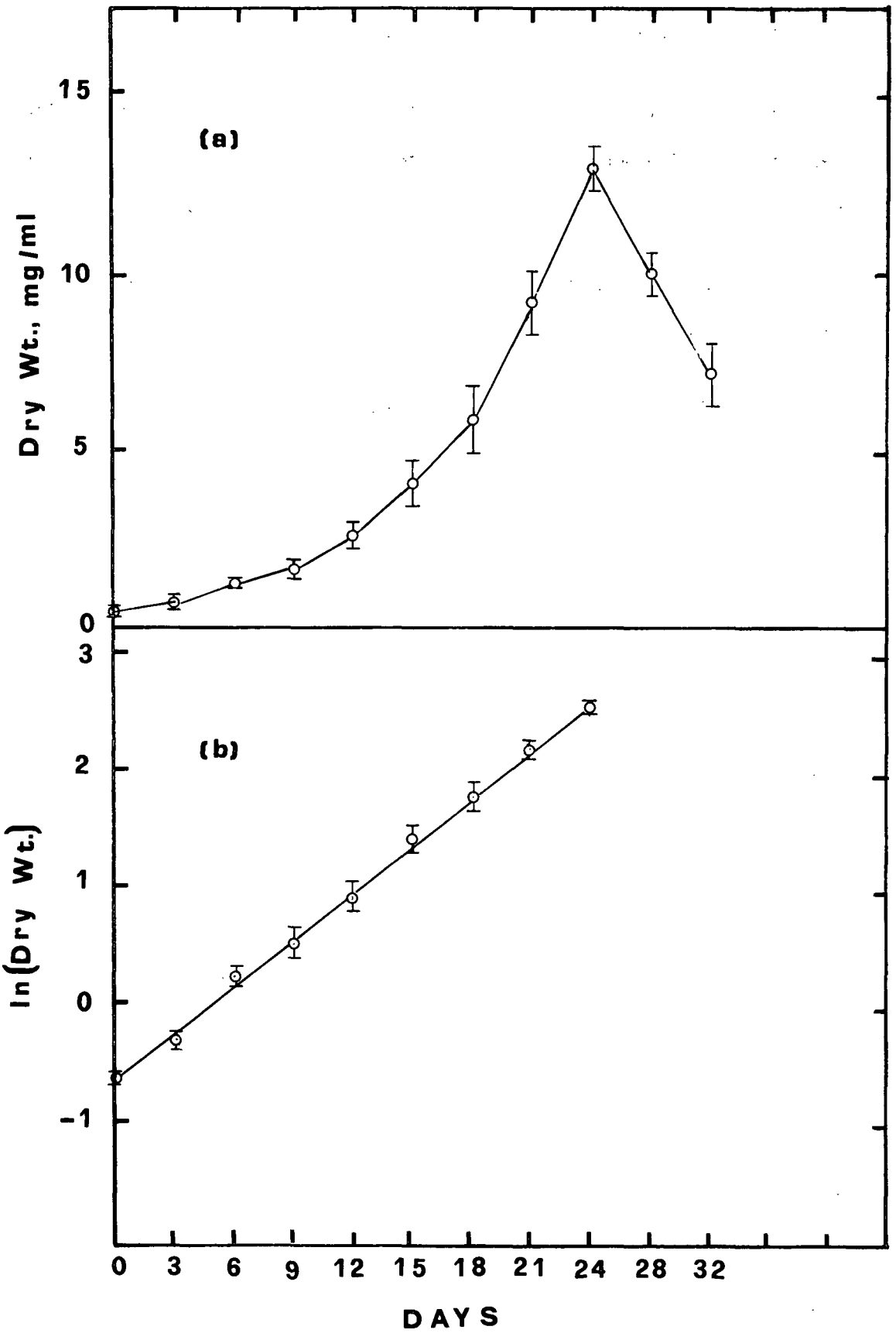

Figure 1A and 1B

Figure 2. Photomicrographs showing stages in the regeneration of cell colonies from loblolly pine protoplasts prepared by treatment F (see Table 1).

(a) Cell wall resynthesis seen at 48 hours of culture by staining with Calcofluor White and examination under ultraviolet light. Bar line represents 120 μm.

(b) A typical small colony visible at 10 days indicating division in protoplast-derived cells. Bar line represents 30 μm.

(c) Numerous colonies in liquid medium visible after 3 weeks. Bar line represents 190 μm.

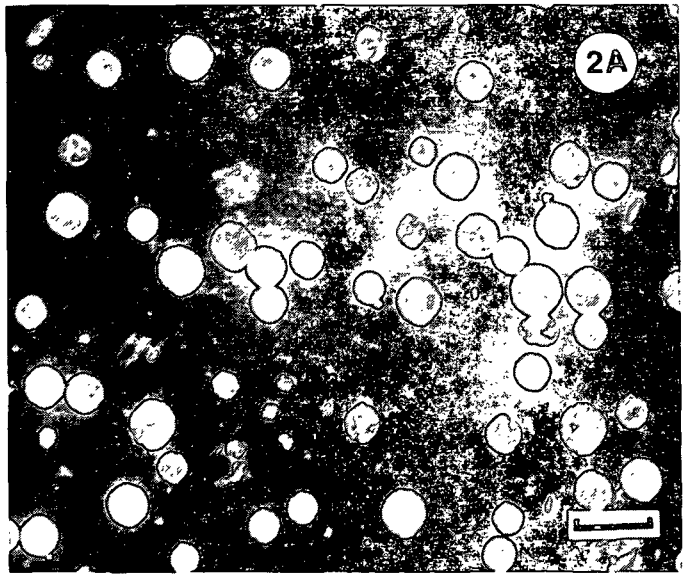

Figure 2A

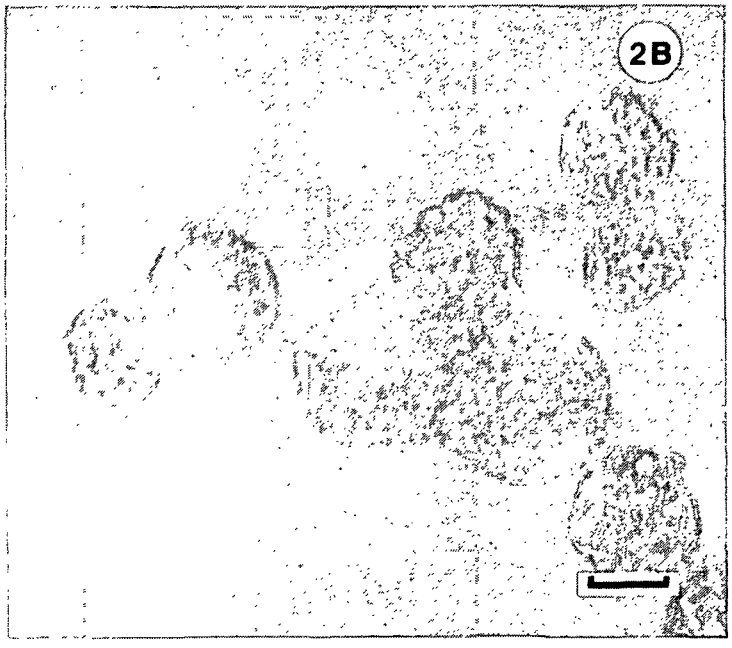

Figure 2B

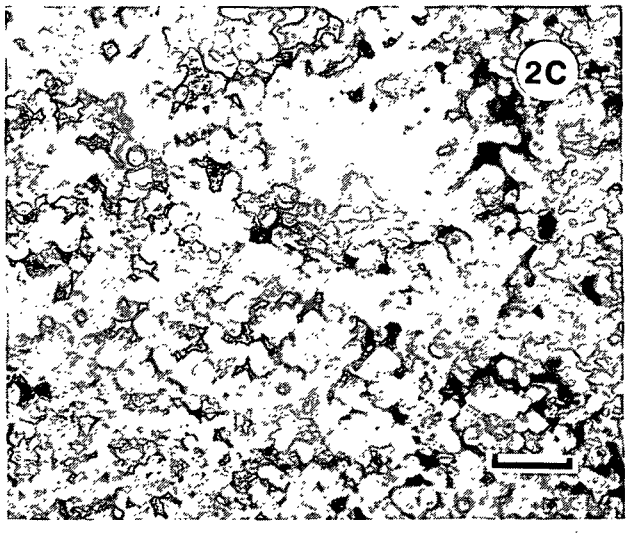

Figure 2C